(54) Title: DETERMINATION AND QUANTIFICATION OF RED BLOOD CELL POPULATIONS IN SAMPLES

(57) Abstract: The invention relates to the detection and determination of erythrocytopathies and hemoglobinopathies. The inven-
tion provides a method for distinguishing between subsets of red blood cells in a sample comprising contacting said sample with at
least a first marker reagent reactive with a first component of a red blood cell and with at least a second marker reagent reactive with
a second component of a red blood cell and determining the reactivity of said marker reagents with said cells.

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TITLE: DETERMINATION AND QUANTIFICATION OF RED BLOOD CELL POPULATIONS IN SAMPLES

The invention relates to the detection and determination of erythrocytopathies and hemoglobinopathies.

The detection of circulating fetal cells in maternal blood samples represents an important area of laboratory support to the obstetrical management of women. Although the concentration of fetal erythrocytes found in the maternal blood circulation during pregnancy is mostly very small and without a clear clinical significance in many cases, substantial hemorrhage may result from a number of causes including fetal or maternal trauma and placental defects (1). The quantification of fetal red blood cells (RBC's) is most commonly used to estimate the degree of feto-maternal hemorrhage (FMH). Either in cases of trauma with suspected placental injury or in the situation of RhD incompatibility between the fetus and the mother for prevention of hemolytic disease of the new-born (HDN) during pregnancy (2, 3). The obstetrical management of women includes the prevention of mother immunization against a foreign fetal cell antigen, and the monitoring of maternal antibody concentration. To prevent an immune response, an immunoprophylaxis based on anti-RhD polyclonal antibodies, is given to the mother at a dose proportional to the estimated count of fetal RBC's present in the maternal blood circulation (4, 5). It is therefore important to be able to at least semi-quantify the relative amount of said cells.

Most clinical laboratories perform FMH estimates on the basis of variations of the slide-based microscopic counting method of acid elution originally described as the Kleihauer-Betke test (6). Although this assay has proven to be clinically useful in the detection of large episodes of FMH requiring maternal treatment with more than the standard dose of Rh immune globulin, it is laborious and suffers from subjectivity and imprecision (7, 8). Besides the experience of the laboratory technicians for interpretation of the results, the test has a tendency to overestimate the size of feto-maternal hemorrhages because maternal HbF containing RBC's or F-cells are counted within the population of fetal cells (9).

Several alternative and more accurate screening methods to detect FMH using flow cytometry have been proposed and described. The first reports investigating the feasibility of using flow cytometry for fetal cell counting primarily relied upon the detection of the human D antigen on the cell surface of RBC's (10, 11, 12, 13). These

5   approaches all demonstrated greater sensitivity and precision than manual methods. However, the use of anti-RhD is applicable only to the clinical situations with Rh or D antigen incompatibility and cannot be utilized in all cases of maternal trauma and suspected FMH. Several other methods for flow cytometric detection of fetal cells in maternal peripheral blood have recently been described. The methods differ in their

10   means of using various cellular fixation and permeabilization steps, usually in combination with the intracellular detection of fetal hemoglobin (HbF) antigen using anti-HbF antibodies.

As an increase of the expression of fetal hemoglobin (HbF) in peripheral red blood cells is also a common feature in hemoglobinopathies comprising genetic disorders of

15   hemoglobin such as sickle-cell disease and beta-thalassemia (14, 15, 16, 17), a method for the detection of HbF in blood cells also finds its use in the diagnosis of hemoglobinopathies other than those related to FHM .

20   The invention provides a method for distinguishing between subsets of red blood cells in a sample comprising contacting said sample with at least a first marker reagent reactive with a first, preferably, antigenic component of a red blood cell and with at least a second marker reagent reactive with a second, preferably antigenic, component of a red blood cell and determining the reactivity of said markers with

25   said cells. In a preferred embodiment, the invention provides a method for distinguishing between and/or quantification of various subsets of erythrocytes in a sample comprising the use of at least two markers reactive with at least two subsets of red blood cells, such as HbF containing erythrocytes (F-cells) or fetal versus adult red blood cells in blood. To diagnose or assess FMH, the invention thus provides for

30   distinguishing between subsets of red blood cells in a sample comprising combining testing for a determinant of essentially fetal cells with a determinant for essentially adult cells. Of course, the various subsets may overlap in that some cells in each subset carry two of the markers used in the method chosen. Said method is most useful to distinguish between subsets of mature erythrocytes, i.e. those that have

matured beyond the nucleated RBC or immature reticulocyte phase. In one embodiment, the invention provides a method for distinguishing between and/or quantification of fetal red blood cells (RBC's) in maternal blood comprising the use of at least two markers reactive with various subsets of red blood cells. In another embodiment, the invention provides a method for distinguishing between and/or quantification of adult HbF containing RBC's in blood comprising the use of at least two markers reactive with various subsets of red blood cells. In spite of the recent reported results of the detection of fetal HbF containing cells in different maternal blood samples, the use of a single parameter does not offer an accurate and reliable quantification of fetal RBC's and maternal F cells. A dual or multiple marker approach has several advantages. Although the use of HbF antigen as single marker allows broad application for fetal red blood cell detection to many clinical situations, the use of anti-HbF by itself provides the possibility of an overestimation of the proportion of true fetal cells in a given HbF population.Use of the intracellular cell marker Carbonic anhydrase (CA) for adult RBC's (18) in combination with HbF should allow for example the clear distinction of fetal red blood cells, from possible interfering maternal F cells that have a lower cellular HbF content and are positive for the CA marker. Small populations of adult erythrocytes containing HbF are found in individuals of any age; these cells have been termed F cells. For people with sickle cell anemia, these cells are functionally quite important because they are capable of transporting and releasing oxygen.

A method is provided for distinguishing between subsets of red blood cells in a sample comprising contacting said sample with at least a first marker reagent reactive with a first component of a red blood cell and with at least a second marker reagent reactive with a second component of a red blood cell and determining the reactivity of said marker reagents with said cells. Suitable cell surface components to be detected are for example: CD71, a type II membrane glycoprotein of 90-95 kDa that exists as a homodimer on most dividing cells including RBC's. The protein plays a critical role in the uptake of iron, through the binding and endocytosis of transferrin, the major iron-carrying protein; GpA, a cell surface sialoglycoprotein of 41 kDa that is exclusively expressed on human erythroid cells and their progenitors. The GpA protein is clinically important in the classification of acute leukemia's; i, a glycosylated Lewis antigen structure expressed on adult T- and B-lymphocytes and fetal lymphocytes and RBC's during the first 8 months of development. For some

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applications, such as flow cytometric detection, it is preferred that at least one of the components to be detected comprises an intracellular component. Suitable intracellular components are for example: HbE, an intracellular hemoglobin protein consisting of 4 protein subunits of approx. 140 amino acids. The embryonic

5   hemoglobin tetramer further consists of different polypeptide chains, epsilon and zeta or epsilon and alpha. The expression of HbE is most prominent in embryonic red blood cells. CA, Carbonic anhydrases (carbonate dehydratase; carbonate hydrolyase) form a large family of genes encoding zinc metalloenzymes of great physiologic importance. As catalysts of the reversible hydration of carbon dioxide, these enzymes

10   participate in a variety of biologic processes, including respiration, calcification, acid-base balance, bone resorption, and the formation of aqueous humor, cerebrospinal fluid, saliva, and gastric acid. CAs are encoded by members of 3 independent CA gene families, i.e., alpha-CA, beta-CA, and gamma-CA. Genes in the alpha-carbonic anhydrase family encode either active carbonic anhydrase isozymes or 'acatalytic'

15   (i.e., devoid of CO_2 hydration activity) carbonic anhydrase-related proteins. Alpha-carbonic anhydrases show extensive diversity in tissue distribution and in their putative or established biologic functions. Some of the alpha-CAs are expressed in almost all tissues (e.g., CA 2), whereas some show a more restricted expression, such as CA 1 in erythrocytes. Erythrocyte carbonic anhydrase has 2 isoenzymes with

20   different amino acid sequences and specific activities. B and C were the original designations for these 2 major forms which later were called CA I (or A) and CA II (or B), respectively. In cells, carbonic anhydrases may reside in cytoplasm, in mitochondria, or in secretory granules, or associate with membranes.

        Such an intracellular component to be detected can be the whole component

25   per se, or can for example be the intra-cytosolic part of a protein or receptor that otherwise projects or extends through the cellmembrane. For intracellular detection of intracellular components, fixation and permeabilization of the red blood cells is required, this advantageously provides rigidity and stability to the erythrocytes to be identified; detecting intracellular antigens in fixed erythrocytes thus allows for less

30   background noise than detecting extracellular antigens in non-fixed cells only. A preferred method is using a combination of two intracellular components (target proteins or antigens) to discriminate between different red blood cell populations of for example fetal, parental or adult origin.

In a preferred embodiment, the invention provides a method wherein said first component consists of hemoglobin F. Hemoglobin (Hb) is an intracellular protein consisting of 4 protein subunits of approximately 140 amino acids. The fetal hemoglobin tetramer further consists of different polypeptide chains, gamma and

5   zeta or gamma and alpha. The expression of HbF is essentially fetal, in that it is most prominent in fetal red blood cells, but is also in low concentrations present in adult red blood cells. HbF is red blood cell specific. Because of the precise discrimination between the cell populations with HbF and another intracellular protein, the invention provides a method for nearly truly quantifying the true fetal cells and not

10   to include or to count possible interfering adult F cells. In another preferred embodiment, the invention provides a method wherein said second component consists of carbonic anhydrase B. Carbonic anhydrase is protein or metalloenzyme with a catalytic activity for CO_2. The expression of CA is essentially or predominantly in adult cells. Much preferred (and further explained in the detailed description

15   herein) is a method wherein said first component consists of hemoglobin F and said second component consists of carbonic anhydrase, especially wherein said carbonic anhydrase is of type I.

A marker reagent used in a method according to the invention can comprise any kind of binding molecule, such as phage derived binding molecules (sometimes

20   also called phage antibodies), but preferred is a method wherein at least one of said marker reagents comprises an antibody. An antibody, in the format of complete Ab or Fab, Fv, sFv, camel derived single chain or other protein structure, is a protein that comprises a so-called light chain and/or a heavy chain, chains, which are each or in combination are responsible for the specific binding of the target antigen. A

25   particularly useful anti-HbF antibody is specific for the gamma protein subunit of hemoglobin F. Antibodies raised against the i antigen are most often specific for this surface protein in the glycosylated form. Monoclonal antibody CD71 is directed towards the transferrin receptor, while anti-GpA antibodies recognize an epitope on the Glycophorin A antigen. Another useful antibody is specific for the epsilon

30   polypeptide chain of embryonic hemoglobin (HbE). Antibodies raised against CA isotypes are specific for the several epitopes present on the different carbonic anhydrases. It is of course preferred for ease of detection that at least two or all of said marker reagents comprise an antibody, each of said antibodies being reactive with a distinct antigenic component of a red blood cell. After fixation and

permeabilization the antibody will enter the cell and bind to the intracellular fixed target protein.

Another type of marker reagent according to the invention comprises a non-proteinaceous molecule, which can bind with high specificity to a target component,
like an inhibitor that can bind to a target enzyme. For example, a sulfonamide inhibitor can be complexed to carbonic anhydrase via the coordination of a primary sulfonamide group to the active site zinc ion (19). Detection of such a marker reagent bound to a target is most easily achieved by using a labeled inhibitor, e.g. a fluorescent derivative of the inhibitor. A useful marker reagent for detecting a target protein according to the invention is a fluorescent inhibitory molecule which labels cells at a low concentration, like in the nanomolar range, and with a short loading time, e.g. within 10 minutes. Also, the efficacy of such an inhibitor should remain essentially unchanged by the fluorescent label. A particularly suitable marker reagent to practice the present invention comprises Bodipy 558/568-modified acetazolamide, a fluorescent inhibitor of CA (20). This modified acetazolamide was used to localize CA in living osteoclasts and can be obtained from Molecular Probes or similar companies. In one embodiment, the invention provides a method to distinguish fetal cells from maternal blood using an antibody against HbF as a first marker reagent and a fluorescent CA inhibitor as a second marker reagent.

In another embodiment of the invention, fetal cells are identified through the use of a marker reagent capable of binding to an intracellular component wherein said component is a nucleic acid. The term nucleic acid, as used herein, refers to either DNA or RNA. For example, nucleic acid probes are useful to detect the presence of a specific nucleic acid sequence in a cell. A variety of methods for specific DNA and RNA measurement using nucleic acid hybridization techniques are well known. For example, a method for evaluating the presence of a certain nucleic acid in a sample involves the use of fluorescent *in situ* hybridization (FISH) technology. Hereto, a sample is contacted with a fluorescent labeled probe having a nucleic acid sequence complementary to the RNA or DNA sequence of interest. Subsequently, hybridization of a probe to a nucleic acid can be measured by a fluorescence detection system e.g. by fluorescent microscopy or flow cytometry. A rapid and sensitive FISH method has been developed to probe RNA or DNA contents of individual cells by flow cytometry. Fixed cells in suspension were hybridized with 5' end-fluorochrome-labeled oligodeoxynucleotides complementary to defined regions of the nucleic acid of

interest and analyzed by flow cytometry (21). The present invention can for instance be practiced using a HbF antibody as a first reagent marker and a 5' end-fluorescein-conjugated oligodeoxynucleotide probe complementary to a stretch of carbonic anhydrase mRNA as a second marker agent. Oligonucleotides labeled with an other

5   fluorochrome, such as coumarin, rhodamine, phycoerythrin, Texas Red and the like, may also be used.

As said, marker reagents, being antibodies, or other binding molecules, are most easily detected when labeled. Useful is detection via a label that comprises a fluorochrome.

10   A fluorochrome to be used can be any of the known molecules used in flow cytometry and or microscopy. Examples of fluorochromes are; protein labels like R-PE, APC, GFP, and chemical labels like, Alexa dyes, Cy dyes, tandem labels between the mentioned dyes, or others. Most of the dyes can be purchased from Molecular Probes or similar companies. Preferred is a method wherein at least two of said marker

15   reagents comprise a fluorochrome, each of said fluorochromes having a distinct emission spectrum. Emission spectra preferably range from 350 to 800 nm. Enzymes (peroxidase, alkaline phosphatase and others) are generally naive or recombinant proteins which can be also attached to antibodies or other marker reagents (labeled) and used to visualize color development with different fluorescent or non-fluorescent

20   substrates like the 2-(5'-chloro-2'-phosphoryloxyphenyl)-6-chloro-4-(3H)-quinazolinone (termed ELF-97 phosphate), tetramethyl benzidine (TMB) and other, which can be purchased from Molecular Probes, Pierce and others.

Preferred is a method as provided herein further comprising determining the reactivity of said marker reagents with said cells by flow cytometry, i.e. by detecting

25   said reactivity of said marker reagents with said cells by detecting fluorescence.

The invention also provides a diagnostic kit suitable for the differentiation of subsets of erythrocytes, said kit at least comprising a first marker reagent reactive with a first component of a red blood cell and a second marker reagent reactive with a second component of a red blood cell, preferably wherein said first component consists

30   of hemoglobin F and/or wherein said second component consists of carbonic anhydrase.

Type I carbonic anhydrase is preferred for reasons explained above. Said marker reagent is preferably an antibody, but can in essence be any binding molecule with the desired binding specificity, said antibody or binding molecule being reactive with

a distinct, preferably intracellular and antigenic component of a red blood cell. Said kit can also comprise the desired fluorochrome or several distinct fluorochromes having a distinct emission spectrum.

The invention also provides a reagent mixture suitable for inclusion in such a

5   kit and suitable for the differentiation of subsets of erythrocytes, said reagent mixture at least comprising a first marker reagent reactive with a first component of a red blood cell and a second marker reagent reactive with a second component of a red blood cell, preferably wherein said first component consists of hemoglobin F and/or wherein said second component consists of carbonic anhydrase. Type I

10   carbonic anhydrase is preferred for reasons explained above.

In the detailed description, we report on a highly accurate and quantitative procedure for the detection of distinct populations of fetal and F cells by flow cytometry that can be routinely performed. The description further illustrates that the use of a dual fluorescent parameter assay, based on the combined detection of fetal hemoglobin

15   (HbF) and carbonic anhydrase (CA), is able to separate true fetal RBC's from interfering maternal F cells.

Furthermore, a fetal antigen, or fetoprotein, also occurs in adults in certain diseases. For example, an increase of the expression of fetal hemoglobin (HbF) in peripheral red blood cells is a common feature in hemoglobinopathies comprising

20   genetic disorders of hemoglobin such as sickle-cell disease and beta-thalassemia. The invention also provides a method for the detection of HbF or another fetoprotein in red blood cells in samples of a subject with a disease such as hemoglobinopathy.

A method according to the invention also finds its use in the field of non-invasive prenatal testing. Prenatal testing includes quantitative or qualitative

25   diagnostics or prenatal evaluation, including determining the sex of the fetus, determining chromosomal, single gene or protein abnormalities, and determining the presence or absence of particular genes, nucleic acids or proteins. To date, there are more than 300 different disorders that can be detected during pregnancy. Some of these are chromosomal abnormalities such as Down syndrome; some are single gene

30   disorders such as cystic fibrosis, Tay-Sachs disease and sickle cell disease. Currently, prenatal diagnosis of chromosome abnormalities requires invasive techniques such as amniocentesis and chorionic villus sampling that carry small but finite risks of fetal loss. A non-invasive approach is to isolate fetal cells from maternal blood by flow sorting followed by genetic interphase analysis with fluorescence in situ hybridization

(FISH). A non-invasive method of prenatal genetic diagnosis requires fetal cell selection from the maternal circulation that allows efficient recovery of fetal cells for FISH analysis. The candidate cell is in general a fetal nucleated red blood cell (NRBC). A current method for enrichment of NRBC's involves initial separation of

5   blood on a density gradient, white cell depletion using a panning technique with a CD45 monoclonal antibody followed by flow sorting based either on selection for CD71+, CD45-, and LDS-751 or gamma-hemoglobin (22). As mentioned before, discrimination between fetal and maternal blood cells on the basis of a single marker is often problematic. Prenatal diagnostics of this type obviously require an optimal

10   separation of fetal cells from maternal cells. Current prenatal diagnostics are often hampered by a poor identification of maternal and fetal subpopulation of cells in a maternal blood sample. In one embodiment of the invention, a method is provided for non-invasive prenatal testing of a fetus, said method comprising the steps of identifying and isolating fetal cells from a maternal blood sample according to the

15   invention and testing said fetal cells.

       The present invention provides a highly accurate and sensitive a method to identify and / or isolate fetal cells that circulate in the maternal blood that is based on a combination of two marker reagents. A dual-color flow cytometric method allows simultaneous detection of these two reagents. Use of a method as provided allows

20   identifying and isolating a population of fetal cells from maternal cells with a high sensitivity and accuracy. In a preferred embodiment, cells in a maternal blood sample are stained with an anti-HbF antibody as a first marker reagent and an antibody reactive with CA as a second marker reagent. Fetal RBC's are recognized by their bright HbF staining in combination with a complete absence of CA expression.

25   Subsequently, this HbF-positive, CA-negative population, corresponding to fetal blood cells, can be separated from maternal cells by flow sorting. Flow sorting may be performed using normal or high-speed multiparameter flow cytometry/cell sorting. Thereafter, isolated fetal cells can be expanded first by *in vitro* culturing or they may be analyzed directly for e.g. chromosomal abnormalities.

30   In another embodiment, an embryonic cell marker is used together with an anti-CA antibody to identify an embryonic RBC population that circulates in a pregnant woman's blood stream. For example, such a procedure can be used to isolate embryonic cells, like erythroblasts, by flow cytometric cell sorting. Embryonic cells may subsequently be subjected to prenatal diagnostics, for example they can be

treated to render the embryonic nucleic acids and/or proteins available for identification and/or amplification. Such a method allows a quantitative or qualitative diagnostic or prenatal evaluation at a very early stage of embryonic development, e.g. as early as six weeks after conception. Use of anti-embryonic

5   epsilon Hb antibodies as marker reagent to identify fetal cells is sometimes preferred. In a preferred embodiment of the present invention, a first marker reagent reactive with embryonic hemoglobin (HbE), for example an antibody directed against the epsilon chain of HbE, is preferably combined with a second marker reagent reactive with CA to detect and / or isolate the rare population of embryonic cells which

10   circulate in a pregnant woman's blood stream. Other suitable embryonic or fetal markers which may be used in the invention are characterized in their temporal expression pattern. Preferably, such a marker comprises an intracellular protein which is highly expressed in embryonal and/or fetal cells whereas its expression is strongly reduced or essentially non-existing after birth. These may include apoptosis-

15   related proteins, such as survivin, transcription factors, like a transcription factor of the basic helix-loop-helix (bHLH) family or a GATA(-like) transcription factor.

       Furthermore, a method provided in the invention finds its application in other clinical situations, such as intra uterine transfusion (IUT). IUT allows blood transfusions and/or medications to be administered to the baby while in the uterus.

20   Blood transfusion by IUT has for example been used over the last three decades to treat fetal anemia caused by Rhesus incompatibility. IUT has become increasingly common and relatively safe since the development of high-resolution ultrasound. The efficacy of IUT can be assessed by taking fetal blood samples and staining fetal cells. The fetal circulation is usually accessed through the placental cord insertion although

25   a free loop or fetal cord insertion can also be used. By fetal cell staining, the percentage of the donor's red cells in the fetal circulation can be determined. The use of a single marker reagent such as an anti-HbF antibody bears the risk of overestimating the proportion of true fetal cells in a given HbF population. As exemplified herein, a method using two marker reagents as provided herein allow for

30   distinguishing of fetal cells from small yet significant populations of adult cells containing HbF, also termed F cells. Thus, the invention provides a method to produce accurate data of true fetal cell frequency for monitoring the efficacy of a clinical procedure like IUT. For example, the method provided permits quantifying fetal cells in a fetal blood sample and calculating the percentage of a donor's red cells

in the fetal circulation. In yet another embodiment of the invention, a method according to the invention finds its use in monitoring abnormal fetal cell trafficking in preeclampsia and other pregnancy related disorders.

As exemplified in the detailed description, the dual flow cytometric procedure
5   provided herein shows an excellent linearity and precision both below and above the clinically important fetal cell frequency of 0.6%. Serial dilutions of mixtures of cord blood and adult blood with a fetal cell frequency range of 0 to 5% were analyzed. Precision was evaluated by performing 10 replicates on each sample within a 5-day period; this consistently demonstrated a coefficient of variation (CV) of < 5%. In a
10   preferred embodiment, the method provided herein has a CV of < 10% for blood samples with >0.1 percent fetal cells, more preferred a CV of <7%, most preferred a CV of <5%.

Flow cytometric analysis allows for the analysis of several different clinical samples, like up to five or even up to seven different test samples, in an hour or less and thus
15   provides a relatively straightforward method amenable to clinical use. The dual flow cytometric method according to the invention provides a technically superior alternative to existing one color flow cytometric procedures. The method provided allows analysis of a large number of cells in a relatively short time compared to other detection techniques such as visual inspection of each test sample by fluorescent
20   microscopy. Also with respect to ease of use in common clinical laboratories, flow cytometry is now preferred over microscopy.

Taken together, the method for distinguishing between red blood cells provided in the invention has an improved performance and is easier and faster in its use compared to existing procedures.

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Figures

30   *Figure 1*

The cytograms of figure 1 demonstrate the population of contaminating autofluorescent WBCs that can be detected and separated by the DNA staining dye LDS 751.

Figure 2

The cytograms of figure 2 are representative of a 10 % of cord blood mixed with normal adult blood and stained with monoclonal antibody directed to HbF. The distributions of fetal HbF+++ cells, adult HbF+ cells and adult HbF- cells are indicated in the different cytograms. The interfering population of adult F cells (HbF+) are difficult to separate from the fetal RBCs in R10 and negative stained adult cells (HbF-) in 4, as demonstrated in Figure 2D.

Figure 3

The cytograms of figure 3 are representative of a 5 % of cord blood mixed with normal adult blood and stained with antibodies directed to HbF and erythrocyte specific CA1. The distributions of fetal HbF+++ cells, fetal HbF++/CA++ cells, adult HbF+/CA++ or F cells, and adult HbF--/CA++ cells, are indicated in the different quadrants of the final cytogram. Figure 3D demonstrates the separation of the interfering adult F cells (R8) from the true fetal cells in R10.

Figure 4

Linearity plot of mixed cord blood experiments. The antibodies, anti-HbF and anti-CA are used to detect the percentage of fetal red blood cells in a background of normal adult blood. The plot shows that the detection and linearity is accurate between at least the 0.1% and 10% of spiked cord blood.

Figure 5

Data of an Intra-uterine-transfusion (IUT) as detected with anti-HbF and anti-CA antibodies. A. The cytograms demonstrate the HbF-containing red blood cell population from a blood sample of the fetus. B. The cytograms demonstrate the detection of the fetal and adult red blood cell populations in the blood of the fetus, after a 50% transfusion with the adult donor blood. C. The cytograms demonstrate the CA- containing red blood cell population present in the adult donor blood sample.

Detailed description

Several alternative and more accurate screening methods to detect FMH using
flow cytometry have been proposed and described. The first reports investigating the
feasibility of using flow cytometry for fetal cell counting primarily relied upon the
detection of the human D antigen on the cell surface of RBC's (10, 11, 12, 13). These
approaches all demonstrated greater sensitivity and precision than manual methods.
However, the use of anti-RhD is applicable only to the clinical situations with Rh or D
antigen incompatibility and cannot be utilized in all cases of maternal trauma and
suspected FMH. Several other methods for flow cytometric detection of fetal cells in
maternal peripheral blood have recently been described (14, 15, 16, 17). The methods
differ in their means of using various cellular fixation and permeabilization steps, in
combination with the intracellular detection of HbF antigen using anti-HbF
antibodies. The flow cytometric anti-HbF approach has several potential advantages
and the HbF antigen allows broad application of flow cytometric fetal red blood cell
detection to many clinical situations. Furthermore, the anti-HbF method provides
good correlation with the standard Kleihauer-Betke test of fetal cell detection,
although with a much higher precision than the manual acid elution assay. However,
our findings with the approach of one cell marker such as HbF indicated that this
single color assay was not precise enough for the enumeration of true fetal cells and
could not rule out the inclusion of a low percentage of false-positive F cells. A small
population of 2 to 8% adult cells containing a low amounts of HbF was observed.
Separation and a clear distinction of both populations of fetal HbF containing cells
and interfering adult F cells with a lower HbF content is very important to produce
accurate data of true fetal cell frequency in maternal blood for FMH assessment or F
cell measurements.

Our findings describe an alternative approach to flow cytometric quantification of
fetal RBC's using antibodies to the intracellular located Fetal Hemoglobin (HbF) and
Carbonic anhydrase (CA), and an optimal intracellular staining technique. The
method or procedure is based on the discrimination between fetal and adult red blood
cells using specific anti-Carbonic anhydrase (CA) polyclonal and monoclonal anti-HbF
antibodies. The HbF antigen/protein is the best marker for the detection of fetal red
blood cells, while the CA antigen/protein is a good marker for adult red blood cell and
is almost absent in fetal red blood cells. The monoclonal and polyclonal antibodies
used in the test are fluorochrome-conjugated for direct and indirect detection of HbF

14

and CA markers in a dual flow cytometry analysis. Both antibody preparations were shown to be specific for HbF and CA respectively using the protocol as described in the material section. No positive cell staining was ever demonstrated without permeabilization of the cells.

5   The preliminary results from the dual-color flow cytometric method were comparable to those in some previous reports describing anti-HbF data. The F cell percentages in whole blood of normal donors obtained during the study were also comparable to those in previous reports. Our findings demonstrate that, by adding a second unique cell marker such as Carbonic anhydrase to the HbF flow cytometric method, it is

10   possible to identify distinct populations of red blood cells. The new method is able to discriminate between true fetal cell and possible false-positive F cells, by shifting the small population of HbF containing F cells from the fetal cell population to the larger population of CA positive adult cells. The complete dual-color staining and analysis of up to 5 samples can be easily performed within 1.5 hour of blood collection. The

15   accurate detection of HbF containing fetal red blood cells in maternal blood circulation will aid to estimate the degree of feto-maternal hemorrhage (FMH) in women during pregnancy, and subsequently in the management of hemolytic disease of the new-born (HDN).
       Finally, the new and sensitive flow cytometric method using a dual fluorescent

20   staining procedure, will accurately identify and quantify both interfering maternal F cells and fetal red blood cell populations. In agreement with other studies (14, 15, 16, 17), it represents a practical and technically superior alternative for the routine measurement feto-maternal hemorrhage compared to the more subjective and manual Kleihauer-Betke test.

25

Materials and Methods
Specimen Collection
Whole blood of 0.5 to 1 ml from normal donors as well as cord blood was collected on EDTA. The samples were usually assayed on the same day or stored at 4 – 8 °C for 1

30   week before testing.

Antibodies and Reagents
Monoclonal antibody NaM16-2F4 (mouse IgG1) specific for the gamma chain of human HbF was previously described (14) and purchased from Bioatlantic (Nantes,

France). Pure antibody preparations were directly conjugated to R-phycoerythrin (R-PE) following standard labeling procedures. Its PE-conjugated IgG1 isotype control was purified and labeled using standard labeling procedures. Polyclonal goat anti-human carbonic anhydrase was purchased from AbCAM while the FITC-conjugated

5   anti-goat was obtained from Sigma. LDS 751, DNA staining dye was purchased from Molecular Probes and stored at 4ºC until use. Newborn calf serum was purchased from Greiner All other reagents were of analytical grade.

Cell Fixation and Permeabilization

10   Ten microliters of whole blood or cord blood were resuspended in to 100 μl of Newborn calf serum (NCS) in PBS, after which the RBC's were fixed with 100 μl of 20% paraformaldehyde in PBS, vortexed for 5 s, and incubated at room temperature (RT) for 30 min. After fixation, the cells were washed once with 2 ml of PBS with heparin and resuspended in 100 μl of PBS with heparin. For permeabilization of the

15   RBC's, 100 μl the fixed cells were mixed thoroughly with 100 μl of 0.3% sodium dodecyl sulfate (SDS) in PBS with heparin and allowed to stand at RT for 3 min. To remove the SDS, the cells were washed twice with 2ml of PBS with heparin and suspended in 1 ml of the same solution.

20   *HbF and CA Detection by Flow cytometry*

For immunophenotyping, 100 μl of the washed cell suspension was then mixed with either 50 μl anti-HbF-PE MoAb diluted at 40 ug/ml in PBS, 50 μl of anti-Carbonic Anhydrase PoAb diluted 1-in-500, and 50 μl of LDS 751; or 100 μl of the washed cell suspension was mixed with 50 μl of LDS 751 and 50 μl of PBS and 50 μl isotype

25   control as a negative control. After an incubation of 15 min. at RT in the dark, cells were washed once with 2 ml PBS containing heparin. The in 100 μl suspended cell solutions were both mixed with 50 ul of anti-Goat IgG-FITC and incubated at RT for 15 min. The cells were washed to remove the secondary labeled conjugate and finally resuspended in 0.5 ml of PBS with heparin, ready for flow cytometry. The isotype

30   control was used instead of the anti-HbF MoAb for a negative control.

Sample acquisition was performed on a Coulter Epics XL MCL flow cytometer (Beckman-Coulter, U.S.A.) The HbF and CA cells were counted by setting the autostop at 50.000 or 100.000 events, with the collection of measures of logFSC and logSSC, and fluorescence signals of logFL1, logFL2 and logFL4 as list mode files.

Data analysis was performed with software (Winlist, Verity Software, Topsham, ME)
on list mode files. The live gate on LDS 751 negative cells was used to exclude
possible interfering nuclei-containing white blood cells. The positive cutoff point was
approximately at 0.5% above negative population of isotype control staining cells.

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Results

A dual-color flow cytometric method results in the simultaneous detection of two
intracellular antigens and provides a convenient and rapid test that can be completed

10   within 1.5 hour of blood collection. The use of paraformaldehyde as fixative reagent
and sodium dodecyl sulfate (SDS) to permeabilize fixed RBC's resulted in low
background staining, negligible HbF leakage, and minimal cell clumping. Following
fixation and permeabilization both cell antigen markers could be detected with high
fluorescence signals, which resulted in a clear distinction between different stained

15   cell populations.
An important aspect of the flow cytometric fetal cell count data is to exclude possible
interfering adult hemoglobin containing RBC's and autofluorescent WBC's present
from the region of fetal cell identification. The induced autofluorescent WBC's can be
readily excluded from the fetal cell population by staining these nuclei-containing

20   cells with a DNA staining dye such as LDS 751 as shown in Figure 1.
The imprecise separation of HbF containing F cells from the true HbF containing
fetal cells is demonstrated in the cytograms of Figure 2. The fact that it is difficult to
set an accurate region on the fetal cells without the interfering F cells, leads to the
overestimation of the true population of fetal HbF containing cells. Especially when

25   the number of fetal cells is low (0.4 − 0.6 %), the true count of fetal cells in a
background of contaminating F cells with variable amounts of HbF is not accurate.
The flow cytometric results of the combination of the two different red blood cell
markers HbF and CA, as presented in Figure 3, demonstrated that most fetal RBC's
with a high HbF content and no CA could be well separated from interfering adult F

30   cells with a lower HbF content but containing high amounts of CA. Adult RBC's with
no HbF were, as expected, only positive for CA. Another small but not unexpected
population of fetal cells, with a high HbF content and lower CA, was detected next to
the fetal cell population and also clearly separated from the F cells. Both the

populations of fetal cells combined together resulted in the true count of fetal RBC's in cord blood and mixtures of cord and adult blood.

The linearity and precision of the dual color flow cytometric method was studied in mixtures of cord blood and whole blood from non-pregnant adults. As shown in Figure

5   4, excellent linearity for the method was observed by serial dilutions of mixtures of cord blood and adult blood (n=10), with a fetal cell frequency range of 0 to 5 %. Precision of the method was determined by performing an analysis on the same preparation of mixtures of cord and adult blood within a 5-day period. The precision of all the samples consistently resulted in a coefficient of variation (CV) of < 5 %.

10 Therefore, the new flow cytometric method demonstrated an excellent assay performance and was observed to be linear and precise both above and below the clinically important fetal cell frequency of approximately 0.6 %. Linearity of measurement was determined for samples consisting of different ratios of cord blood mixed with normal adult blood in the range of 0.0 – 10 % positive cord blood cells

15 (HbF/CA). Nine months old cord blood consists of 85 % fetal RBC's. The linear regression method was used to plot the know expected values versus the observed values for the percent of double labeled fetal cells determined by the double fluorescent cytometric method.

Figure 5 shows that the method provided herein for distinguishing between subsets

20 of red blood cells in a sample is also advantageously used to monitor the efficacy of intra-uterine blood transfusion. The cytograms demonstrate a clear distinction between red blood cell populations in an adult donor blood sample and in a fetal blood sample before and after a transfusion with the donor blood.

25   Our studies indicate that the use of a second antibody for the detection of CA, preferably in combination with the HbF marker or with another determinant of essentially fetal cells, provides a more detailed discrimination of the different fetal and adult RBC populations and results in an improvement in the ability to determine fetal RBC frequency.

30

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Claims

1.   A method for distinguishing between subsets of red blood cells in a sample
comprising contacting said sample with at least a first marker reagent reactive with
a first component of a red blood cell and with at least a second marker reagent
reactive with a second component of a red blood cell and determining the reactivity of
said marker reagents with said cells.

2.   A method according to claim 1 wherein at least one of said components
comprises an intracellular component.

3.   A method according to claim 2 wherein said first component consists of
hemoglobin F.

4.   A method according to claim 2 wherein said second component consists of
carbonic anhydrase.

5.   A method according to claim 2 wherein said first component consists of
hemoglobin F and said second component consists of carbonic anhydrase.

6.   A method according to anyone of claims 1 to 5 wherein at least one of said
marker reagents comprises an antibody.

7.   A method according to claim 6 wherein at least two of said marker reagents
comprise an antibody, each of said antibodies being reactive with a distinct antigenic
component of a red blood cell.

8.   A method according to anyone of claims 1 to 7 wherein at least one of said
reagents comprises a fluorochrome.

9.   A method according to claim 8 wherein at least two of said marker reagents
comprise a fluorochrome, each of said fluorochromes having a distinct emission
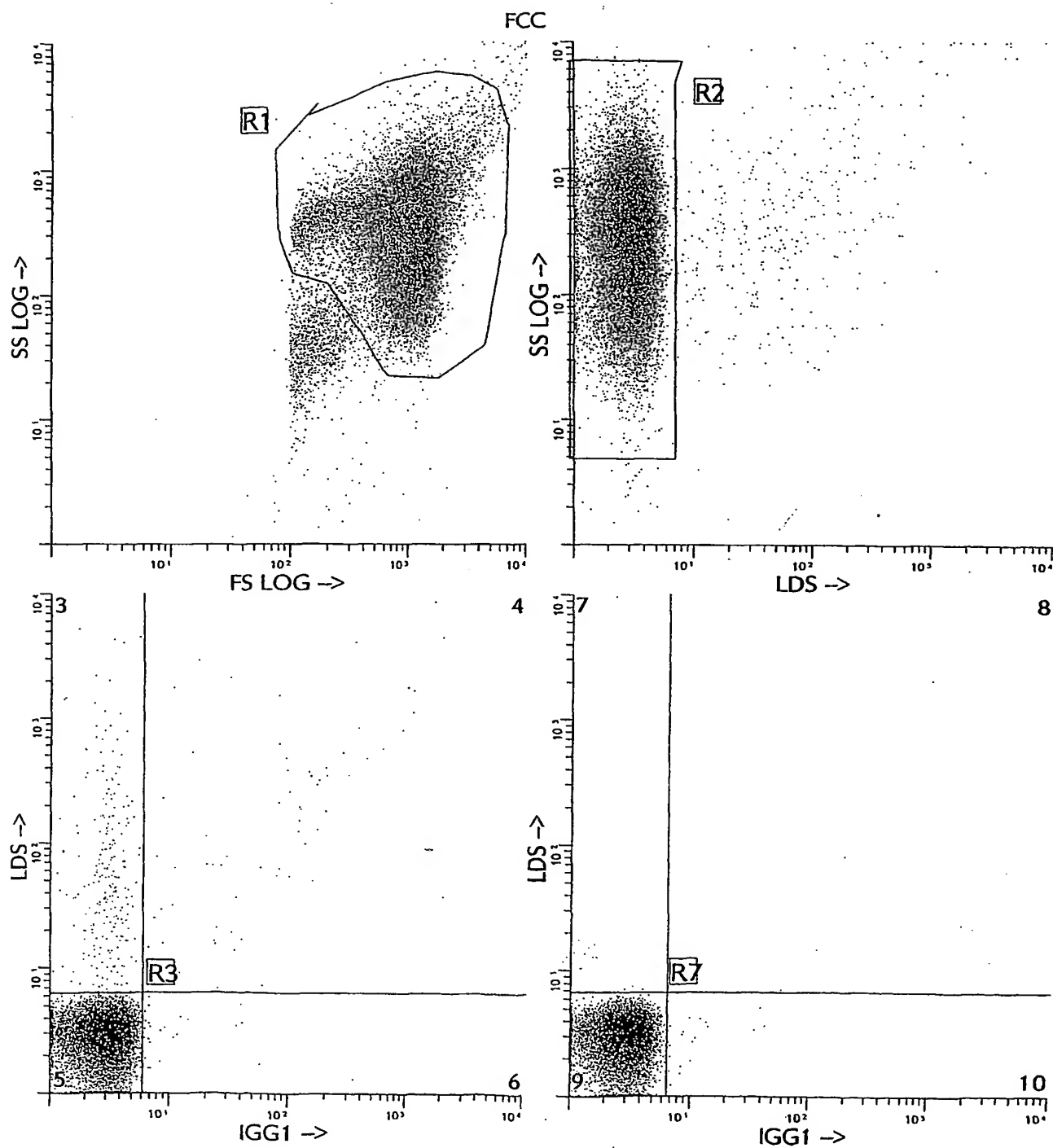spectrum.

10.   A method according to anyone of claims 1 to 9 further comprising determining the reactivity of said marker reagents with said cells by flow cytometry.

11.   A method according to anyone of claims 1 to 10 further comprising determining the reactivity of said marker reagents with said cells by detecting fluorescence.

12.   A diagnostic kit suitable for the differentiation of subsets of erythrocytes, said kit at least comprising a first marker reagent reactive with a first component of a red blood cell and a second marker reagent reactive with a second component of a red blood cell.

13.   A kit according to claim 12 wherein the reactivity of said marker reagents with said cells is determined by flow cytometry.

14.   A kit according to claims 12 or 13 wherein said first component consists of hemoglobin F.

15.   A kit according to claims 12 or 13 wherein said second component consists of carbonic anhydrase B.

16.   A kit according to claims 12 or 13 wherein said first component consists of hemoglobin F and said second component consists of carbonic anhydrase B.

17.   A reagent mixture suitable for use in the differentiation of subsets of erythrocytes, said mixture at least comprising a first marker reagent reactive with a first component of a red blood cell and a second marker reagent reactive with a second component of a red blood cell.

18.   A method to monitor the efficacy of intra uterine transfusion (IUT), said method comprising quantifying fetal cells in a fetal blood sample according to anyone of claims 1 to 11 and calculating the percentage of a donor's red cells in the fetal circulation.

19.   A method for non-invasive prenatal testing of a fetus, said method comprising the steps of identifying and isolating fetal cells from a maternal blood sample according to anyone of claims 1 to 11 and testing said fetal cells.
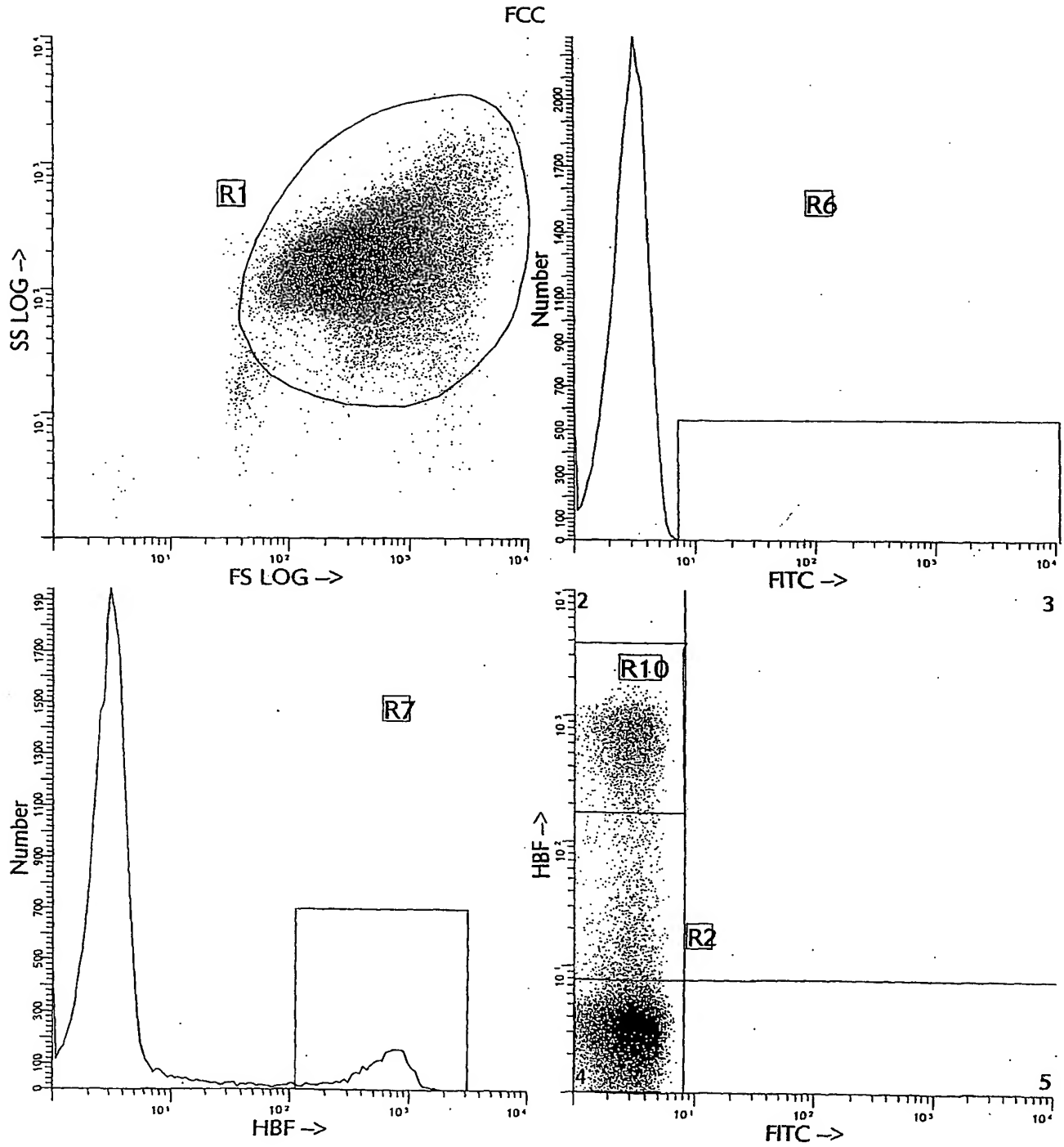
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Fig. 1
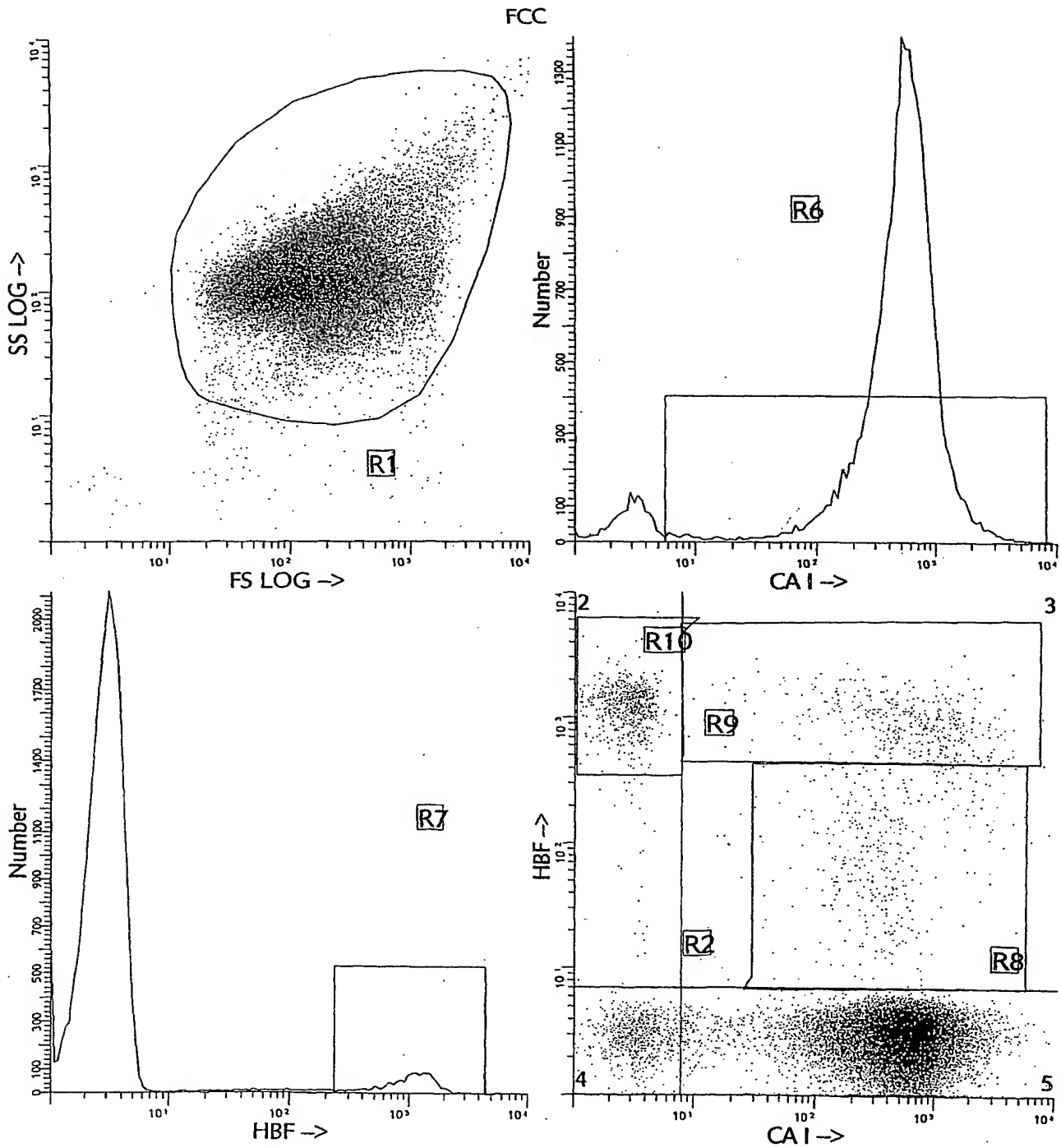
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Fig. 2
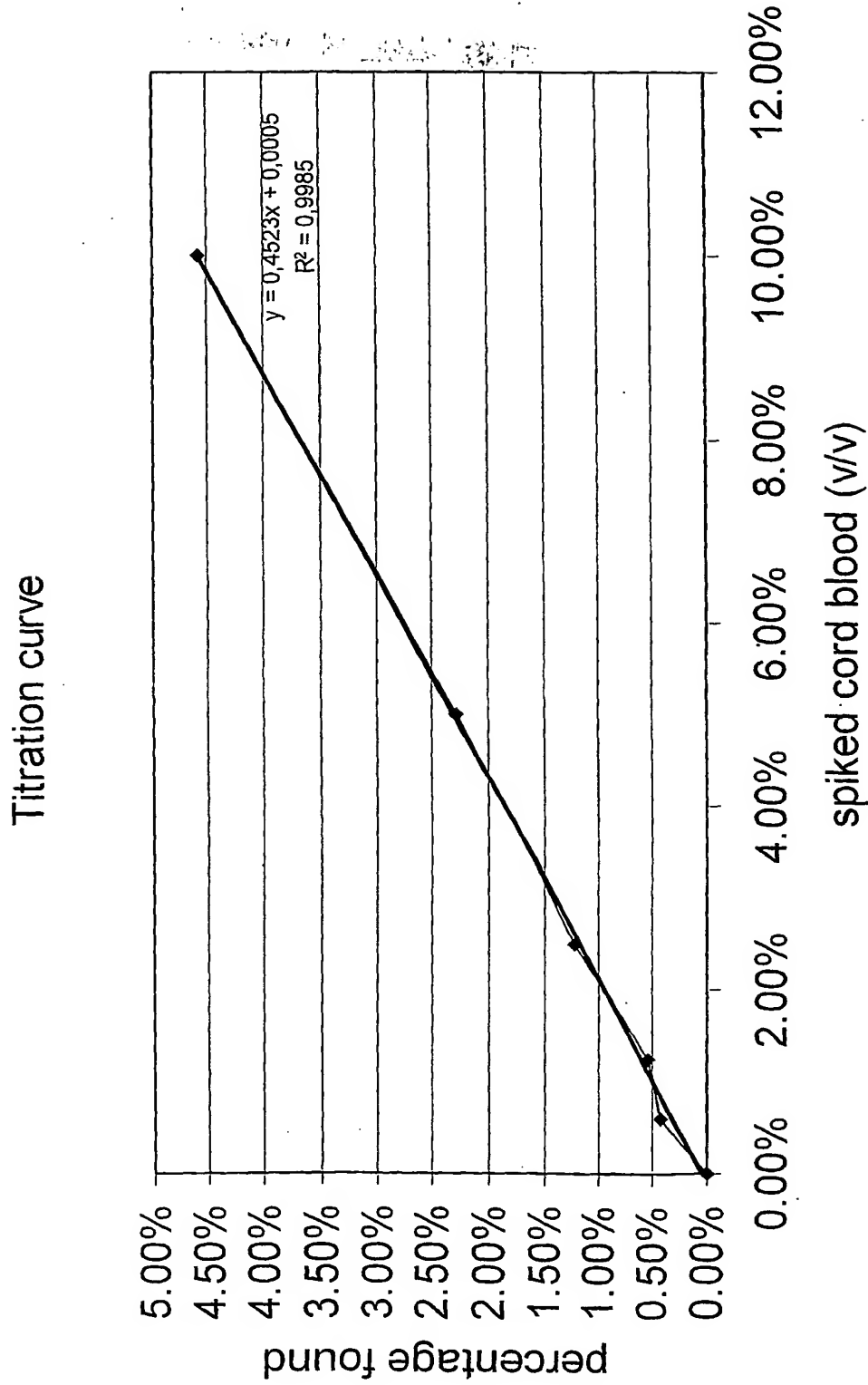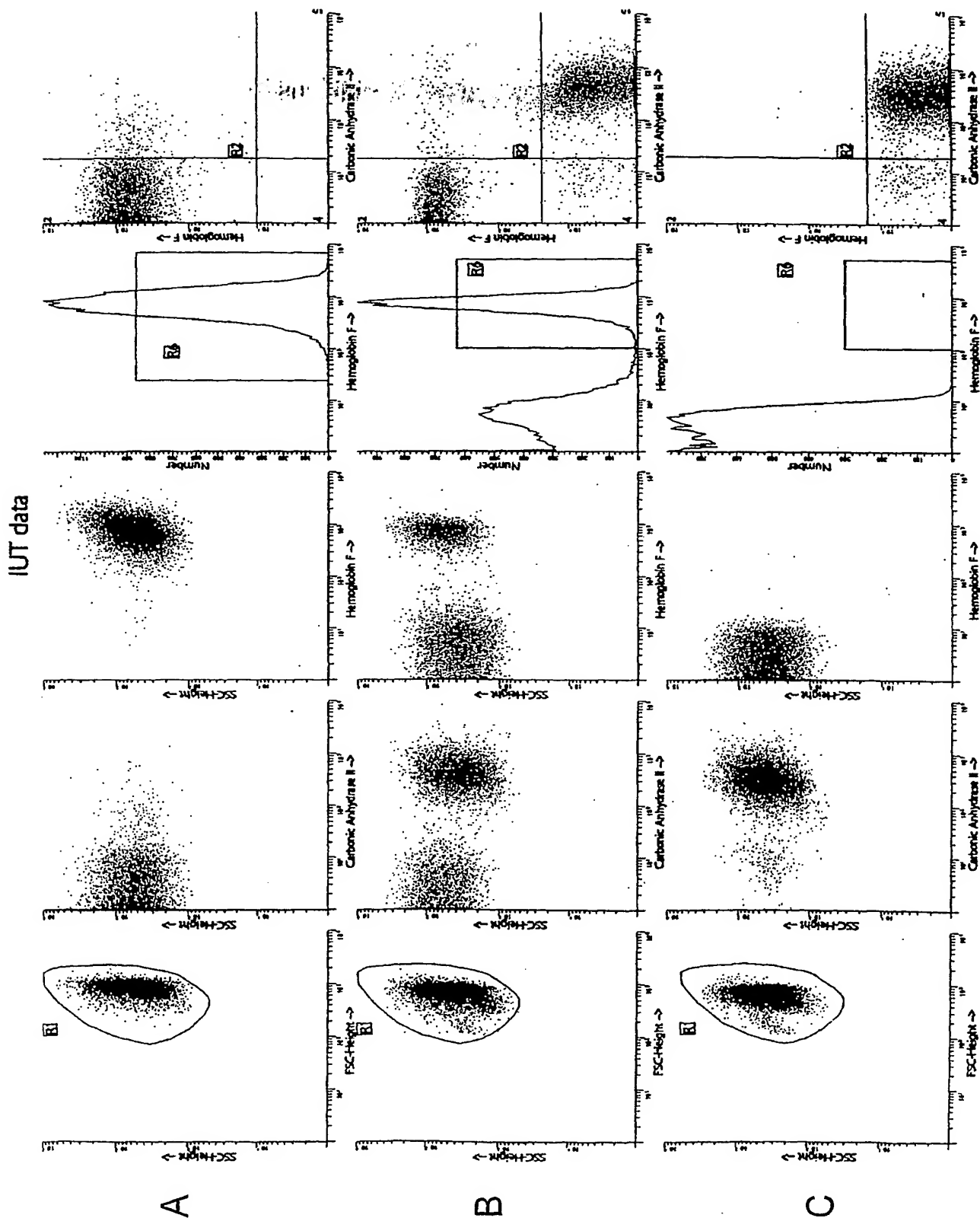
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Fig. 3

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Titration curve

$y = 0,4523x + 0,0005$
$R^2 = 0,9985$

Fig. 4

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Fig. 5

IUT data

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